ANTI-PETASIN ANTIBODIES, METHODS FOR THE PRODUCTION THEREOF AND THEIR USE

Description

The invention relates to anti-petasin antibodies for detecting petasin or petasin protein conjugates in physiologic liquids which do not show any cross reactivity to derivatives, structural analogues or metabolics of petasin, methods for producing them by means of immunization by petasin derivatives which are preferably coupled to a carrier molecule and to their use and a test kit.

Petasin, a component of butterbur extracts is – as is known – an ester consisting of petasol and angelic acid which already for a longer time has been used as vegetable spasmoanalgesic for combatting spasms of the gastrointestinal tract, in particular ureteral colics, spastic bronchititis and migrain and antiphlogistically (B. Debrunner et al.; Pharm. Acta Helv. 72, 359-380 (1998). In addition, an antitumour effect is ascribed to petasin drugs (B. Meier et al., Hagers Handbuch der pharmazeutischen Praxis (Manual of pharmaceutical practice), 5th edition, p. 81-105, Springer-Verlag (1994)). In the mean time also latest findings relating to the effects on the biosynthesis of leukotrienes are available (D. Pichl et al., Planta Medica, 60, 318-322 (1994)).

After peroral application of petasin drugs only concentrations in the range of a few ng/ml are to be expected in body fluids of healthy probands. Owing to this background biological, physical and chemical methods of detection applied for characterizing the drug itself may not be used for quantifying petasin in body fluids. Even most up-to–date analytical methods as the HPLC usually applied are not sufficiently sensitive or not suited owing to requiring much time for large numbers of samples.

That is why the invention was based on the task to provide methods of detecting petasin, in particular suitable methods with a high sensitivity and specificity which allow a good bioavailability for the desired pharmacokinetic investigations.

According to the present invention immunochemical methods of detection meet the requirements for sensitivity and specificity thus not requiring an additional extraction or

through an activated hydrazide dextran with the carboxyl group being preferably inserted with carboxymethylhydroxyamine forming oxime.

- Derivatives of petasin of the general formula I where the double bond in positions 11,12 is brominated and coupled to bovine serum albumin activated by means of a Traut's reagent.
- Derivatives of petasin of the general formula I where angelic acid has been split off and the remaining petasol has been coupled to a carrier through chloroformic acid ester.

The anti-petasin antibodies thus produced do not show any cross reactivity to derivatives, structural analogues or metabolites of petasin and are used for detecting petasin or petasin-protein conjugates in physiologic liquids with either petasin, petasin protein conjugates or the anti-petasin antibodies showing preferably a marker. The reactants are preferably available in a homogeneous solution.

Enzymes, fluorescent dyes, radioisotopes or redoxactive compounds are used as markers.

Petasin bound to antibodies is optically, electrochemically, fluorimetrically or radiochemically detected, preferably optically by means of colour reagents or chromatographically.

In one variant either anti-petasin antibodies, the petasin to be detected or the petasin protein conjugates are bound to a solid phase with a washing process taking place between the reaction steps.

If necessary, the solid phase is chemically activated, with binding of the anti-petasin antibodies, the petasin to be detected or the petasin-protein conjugate to it being effected adsorptively or covalently. Polystyrene is preferably used as solid phase.

In addition, the solid phase may have a differing geometric shape, thus e.g. the shape of a microtitration plate, a tube or a spherical or planiform shape.

Furthermore, the invention relates to a test kit for detecting petasin in physiologic liquids comprising

anti-petasin antibodies,

a solid phase of polystyrene,

washing solution,

dilution buffer,

marked petasin or a marked anti-species antibody,

a marker-specific detection system, preferably an enzyme substrate.

Hereinafter the invention is explained in greater detail by means of examples.

Examples

A) Production of immunogenes

Petasin oxime:

10 mg (3.3×10^{-5} mol) of petasin are to be dissolved in 5 ml of ethanol, 15 mg (6.8×10^{-5} mol) of carboxymethoxylamine hemihydrochloride (Sigma-Aldrich) are to be added and 5 M sodium hydroxide solution are to be added drop by drop until a pH of 12 will be reached. The batch is refluxed for 4 h, evaporated to dryness on a water bath, washed with 2 M hydrochloric acid and dissolved in a mixture of 1 ml of dioxan and 2 ml of DMSO and stored at -70°C.

Thin-layer chromatography: R_f value (silica gel G60, chloroform) = 0.42 (petasin: 0.16).

Oxime is formed as sole reaction product.

Petasin oxime bovine serum albumin:

32 mg (4.8×10^{-7} mol) of bovine serum albumin (BSA) are to be dissolved in 4 ml of PBS (solution A).

7 mg (1.8×10^{-5} mol) of petasin oxime, dissolved in 1 ml of dioxan/DMSO = 1:2 (v/v), 16 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) are to be added and while being stirred incubated for 30 min. at room temperature (solution B).

Solution B is added drop by drop to solution A, stirred for 6 h at room temperature, subsequently dialysed at 4°C against 3x0.5 l of PBS (0.01 M phosphate, 0.15 M NaCl, pH = 7.4) and stored at –70°C.

Petasin-dextran proteins:

7 mg ($1.8x10^{-5}$ mol) of petasin oxime, dissolved in 1 ml of dioxan/DMSO = 1:2 (v/v) are added drop by drop to 32 mg of bovine serum albumin ($4.8x10^{-7}$ mol) or fibrinogen in 4 ml of PBS and 0.5 mg ($1.5x10^{-4}$ mol hydazide groups) of activated hydrazide dextran (Pierce, Code 20900) are to be added. Thereupon, 16 mg of 1-ethyl-3-(3-dimethylaminopropyl(carbodiimide (EDAC) are to be added and the mixture is to be incubated for 4 h at room temperature. Thereupon, a dialysis is carried out at 4°C against 3x0.5 l of PBS (0.01 M phosphate, 0.15 M NaCl, pH = 7.4). Storage is effected at –70°C.

Bromopetasin bovine serum albumin:

Bromating of petasin:

10 mg ($3.1 x10^{-5}$ mol) of bromine in 1 ml of dichloromethane, dissolved in 3 ml of dichloromethane, are added drop by drop with swirling to 5 mg of ($3.3x10^{-5}$ mol) petasin. Thereupon, the batch is evaporated to dryness on a water bath and taken up in 1 ml of DMSO. Thin-layer chromatography: R_f value (silica gel G60, chloroform) = 0.51 (petasin: 0.16).

Thiolation of bovine serum albumin:

40 mg ($6x10^{-7}$ mol) of bovine serum albumin is to be dissolved in 1 ml 0.1 M of phosphate buffer, pH = 8.0, and 20 mg ($1.4x10^{-4}$ mol) of 2-iminothiolan hydrochloride (Traut's reagent) are to be added and incubated for 40 min. at room temperature. Subsequently, with the aid of a column filled with Sephadex G25 (1x10 cm) an buffer exchange is carried out against 0.1 M phosphate buffer pH = 7.2. 4 mg ($8.4x10^{-6}$ mol) of bromopetasin are added while being stirred for dissolving the thiolated protein and an incubation is effected for 3 h at room temperature, thereupon a dialysis is carried out at 4°C against 3x0.5 l of PBS (0.01 M phosphate, 0.15 M NaCl, pH = 7.4).

Petasol bovine serum albumin:

0.7 mg ($2.9x10^{-6}$ mol) of petasol are dissolved in 200 µl of dried dioxan/DMF = 1:1 (v/v), 2 mg ($7.9x10^{-6}$ mol) of 5-norbornene-2,3-dicarboximidyl chloroformic acid ester and 4 mg ($3.3x10^{-5}$ mol) of 4-dimethyl amino pyridine are added and the mixture is incubated for 1 h at room temperature excluding atmospheric humidity. Thereupon, this solution is added drop by drop with stirring to 10 mg ($1.5x10^{-7}$ mol) of bovine serum albumin, dissolved in 0.5 ml of PBS and incubated for 2 h at room temperature. Thereupon, it is dialysed at 4°C against 3x0.5

l of PBS (0.01 M phosphate, 0.15 M NaCl, pH = 7.4) and protein conjugate is stored at – 70°C.

B) Production of anti-serum

Immunization is effected in rabbits as primary injection by subcutane and intramuscular injection with always 3 mg of petasin-BSA in a complete Freund's adjuvant. The secondary injection is effected four weeks after the primary injection. After further two weeks the first booster injection is effected, a second one is carried out twelf weeks after the beginning of immunization in an incomplete Freund's adjuvant. About eight weeks after starting immunization the first blood sample is taken which is supplemented by a further one after four weeks. Exsanguination is carried out after 16 weeks.

The antiseruma obtained are subjected to a titre determination for specific anti-petasin antibodies by means of an enzyme immunoassay where petasin ovalbumin is bound to the surface of microtitration plates. The antiseruma to be examined and the normal sera of the rabbits are subsequently incubated in a dilution series with the immobilized petasin. The bound antibodies are detected by incubation with a goat-anti-rabbit immunoglobuline enzyme conjugate (peroxidase) and subsequent visually evaluable substrate reaction.

C) Enzyme immunoassay

Petasin ovalbumin:

4 mg of EDAC are added to 0.3 mg ($8x10^{-7}$ mol) of petasin oxime, dissolved in 100 µl of dioxan/DMSO = 1:2 (v/v) and incubated for 30. min. at room temperature. Subsequently, the batch is put into a solution of 5.5 mg ($1.2x10^{-7}$ mol) of ovalbumin in 3 ml of PBS, incubated for 2 h at room temperature while being stirred and subsequently for 16 h at 4°C. The reaction mixture is dialysed at 4°C against 3x0.5 l of aqua bidest. and the protein conjugate is stored at –70°C.

Coating:

Petasin ovalbumin is adsorptively bound to polystyrene microtitration plates in a concentration of 5 mg/l in 0.1 M carbonate buffer, pH = 9.5, (100 µl/well) for 16 h at 4 °C and thereupon sucked off. After washing it two times with 300 µl/well washing buffer (PBS, 0.1

% Tween 20) it is blocked for 2 h at room temperature with 150 µl/well blocking solution (0.6 % gelatine, 0.02 % sodium acid in PBS) and subsequently washed three times with washing buffer.

Execution of the test:

50 µl of the serum sample to be tested or the respective standard (1:4 dilution in a sample buffer (PBS, 1 % BSA, 0.1 % Tween 20, 0.01 % thiomersal) and 50 µl of an optimized anti-serum dilution in a sample buffer are simultaneously incubated with shaking for 1 h at room temperature. Subsequently, the microtitration plate is washed three times with 300 µl/well of washing buffer and incubated for 30 min. at room temperature with 100 µl of anti-rabbit immunoglobulin peroxidase conjugate, diluted in sample buffer, and once more washed as above. Thereupon, it is incubated for 10 min. with 100 µl of a substrate solution ready for use (3.3', 5.5'-tetramethyl benzidine) per well and the reaction is stopped by adding 100 µl/well of 0.5 M sulphuric acid. The evaluation is carried out at 450 nm in a microtitration plate reader.

Description of indication

Plant extracts obtained by means of special methods from leaves or rhizomes of *Petasites hybridus L.* may inhibit the 5-lipoxygenase. Thus, the arachidonic acid cascade is effectively interrupted in the case of allergic inflammations. In particular, the release of leukotriene from endogenic cells stimulated in the case of inflammations is stopped, inter alia also from eosinophilic and neutrophilic leukocytes.

Thus, such plant extracts are potential candidates for the therapeutic use in the case allergic inflammations such as allergic rhinitis, asthma, atopic dermatitis, colitis ulcerosa etc. First clinical experience proves the therapeutic efficiency of this plant extract in the case of allergic rhinitis. A prophylactic use of the extract in the case of selected forms of migrain gave also indications to its efficiency.

In addition to detecting the plasma level required for the efficiency for relevant components of the extract, e.g. petasin, the knowledge of the pharmacokinetics of such relevant components are urgently required for a medical use of the plant extract. With the anti-petasin antibodies according to the invention in an enzyme immunoassay a secure detection of petasin in the blood in the lower ng range is achieved. The result of a pharmacokinetic examination enclosed proves impressively its usability.

In a 1st phase of the clinical test for determining the pharmacokinetic parameters of tablets containing butterbur extract single oral administrations of 2 or 4 tablets to 24 clinically healthy men at the age between 18 and 40 years were evaluated.

The open, cross-over test was chosen as method, single administration of each dose in a randomized order with an interval of at least 7 days between the administrations.

Efficiency:

Model-independent pharmacokinetic parameters for petasin

Statistical methods:

ANOVA, $ANOVA_{log}$, Wilcoxon-Mann-Whitney test, Wilcoxon-sign order test

Summary – conclusions

Results:

Petasin serum concentration (ng/ml)

	after administering 2 tablets						after administering 4 tablets					
Zeit p.a.(h)	N	Mean	S.D.	Min.	Median	Max.	N	Mean	S.D.	Min.	Median	Max.
0	0	0,0		0,0		0,0	0	0,0		0,0		0,0
0,25	9	2,8	1,8	1,1	2,2	5,5	13	4,2	3,7	1,0	3,2	14,9
0,5	20	7,6	5,8	1,5	5,9	23,3	21	21,2	24,9	1,3	13,7	96,2
0,75	20	11,9	5,7	4,0	11,0	23,8	19	28,7	22,5	2,5	23,0	91,8
1	20	15,6	7,2	4,0	14,7	29,3	20	36,6	23,0	7,8	38,1	100,0
1,167	20	21,0	16,1	5,6	15,3	62,9	20	47,3	29,1	7,5	43,6	100,0
1,5	20	19,3	12,0	5,1	16,1	47,3	19	40,8	22,3	12,2	32,4	90,7
1,833	20	18,2	11,3	7,8	14,7	44,3	20	32,0	20,1	13,8	26,8	100,0
2,167	20	16,3	8,3	7,3	14,5	31,7	20	28,9	15,0	11,4	27,5	76,1
2,5	20	13,6	6,4	5,9	10,2	26,6	19	24,3	10,7	8,4	26,1	40,9
3	20	8,8	4,1	3,1	7,7	18,3	20	17,9	10,0	7,2	14,6	49,0
4	20	4,5	2,6	1,7	5,2	11,2	20	9,5	5,2	2,9	8,1	20,8
5	20	4,1	2,3	1,5	3,4	8,8	21	12,4	16,5	3,2	7,3	81,4
6	18	3,2	1,7	1,2	3,1	8,1	21	5,8	3,8	1,6	5,0	14,7
8	18	1,9	0,8	1,0	1,6	4,2	19	3,9	3,1	1,4	3,1	15,7
12	13	1,6	0,5	1,1	1,4	2,9	18	2,7	1,0	1,3	2,5	5,1
24	6	1,5	0,5	1,1	1,3	2,3	10	1,3	0,4	1,0	1,0	2,3

Values below the detection limit (1 ng/ml) are equated with 0.

Model-independent pharmacokinetic parameters (± S.D.)

parameter/dosage	2 tablets	4 tablets
C_{max} (ng/ml)	25,5	58,1
SD	±14,8	± 26,7
t_{max} (h)	1,616	1,614
SD	±0,499	± 0,926
$AUC_{0-t(last)}$ (ng/ml*h)	65,30	151,15
SD	± 35,61	± 68,21
$AUC_{0-\infty}$ (ng/ml*h)	79,68	168,22
SD	± 42,27	± 73,43
AUC_{Rest} (%)	18,3	10,8
SD	± 7,9	± 4,9
$T_{1/2}$ (h)	7,155	7,618
SD	± 4,611	± 3,338
MRT (h)	7,32	6,74
SD	± 3,74	± 2,47

Security parameters:

No significant and clinically relevant modifications of the haematological and clinical.chemical laboratory parameters

Undesired events:

Undesired events did not occur.

Conclusions:

- The resorption takes quickly place depending on the dose.
- Both dosages shall be regarded to be equal as to their bioavailability.

Mathematical-statistical evaluation

1. Pharmacokinetic and statistical calculations

The serum levels of petasin measured were the basis of the evaluation.

2. Model-independent pharmacokinetic parameters

The averages and standard deviations (SD) of the pharmacokinetic parameters have been summed up in Table 1.

Parameter/dosage	2 tablets	4 tablets
C_{max} (ng/ml) ± SD	25,5 ±14,8	58,1 ± 26,7
t_{max} (h) ± SD	1,616 ±0,499	1,614 ± 0,926
$AUC_{0\text{-}t(last)}$ (ng/ml*h) ± SD	65,30 ± 35,61	151,15 ± 68,21
$AUC_{0\text{-}\infty}$ (ng/ml*h) ± SD	79,68 ± 42,27	168,22 ± 73,43
AUC_{Rest} (%) ± SD	18,3 ± 7,9	10,8 ± 4,9
$t_{1/2}$ (h) ± SD	7,155 ± 4,611	7,618 ± 3,338
MRT (h) ± SD	7,32 ± 3,74	6,74 ± 2,47

The dose-dependent parameters C_{max} and AUC are nearly proportional to the dose, the deviations of the averages of all other parameters are nearly identical considering the standard deviations determined.

The big standard deviations have to be regarded as an expression of interindividual differences, notably of the speed of resorption, distribution and metabolism of petasin. Thus, after administering the low dose a petasin serum level above the determination limit of the analyzing method has not been detected at no time.

The calculation of the relevant bioavailability (calculation of the dose-corrected quotient of the pharmacokinetic parameters with a 90 % confidence interval) of the dose of 4 tablets compared with a dose of 2 tablets of the test medication shows:

Table 2

Comparative bioavailability

Butterbur 4 tablets versus butterbur 2 tablets

		C_{max}	T/R (%)	$AUC_{0-\infty}$	T/R (%)
adoption of distributions	Statistical method	point esti-mation	Conf.interv. from .. .to		Conf.interv. from... tos
normal distr	ANOVA (x-Over)	113,5	91,9 ... 135,0	106,2	86,5 ... 126,0
log-normal distrib.	ANOVA log (x-Over)	114,9	94,7 ... 139,5	109,1	92,5 ... 128,6
distribution-free	Wilcoxon-Mann-Whitney Test	113,5	87,7 ... 141,8	101,0	87,5 ... 121,3
	Wilcoxons sign-order-test	111,4	93,4 ... 135,4	104,5	90,8 ... 122,3

In the framework of the limits between 70 and 142.9 % for C_{max} and between 80 and 125 % for AUC usually accepted in bioavailability tests the availability of both dosages is to be regarded as equal.

Table 3

Groups stastistic of the petasin concentration (ng/ml) in serum after administering 4 tablets

Zeit p.a.	N	Mean	S.D.	Min.	Median	Max.
0	0	<1			<1	
0,25	13	4,2	3,7	1,0	3,2	14,9
0,5	21	21,2	24,9	1,3	13,7	96,2
0,75	19	28,7	22,5	2,5	23,0	91,8
1	20	36,6	23,0	7,8	38,1	100,0
1,167	20	47,3	29,1	7,5	43,6	100,0
1,5	19	40,8	22,3	12,2	32,4	90,7
1,833	20	32,0	20,1	13,8	26,8	100,0
2,167	20	28,9	15,0	11,4	27,5	76,1
2,5	19	24,3	10,7	8,4	26,1	40,9
3	20	17,9	10,0	7,2	14,6	49,0
4	20	9,5	5,2	2,9	8,1	20,8
5	21	12,4	16,5	3,2	7,3	81,4
6	21	5,8	3,8	1,6	5,0	14,7
8	19	3,9	3,1	1,4	3,1	15,7
12	18	2,7	1,0	1,3	2,5	5,1
24	10	1,3	0,4	1,0	1,0	2,3

Values below the detection limit (1 ng/ml) correspond to 0.

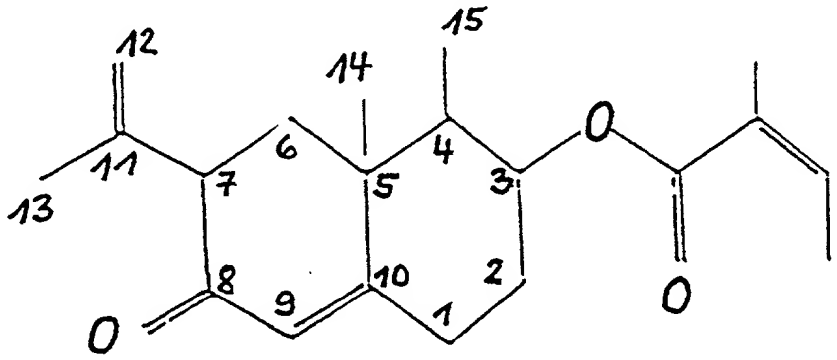
From the attached figure there may be seen that the medium maximum petasin concentration (C_{max}) has nearly doubled after administering double the dose. The medium time of reaching the maximum serum level (t_{max}) remains constant.

concentration of the sample advancing the proper determination as it is required when applying chromatographic methods.

It was possible to accomplish this task by providing anti-petasin antibodies which, in particular, do not show any cross reactivity to derivatives, structural analogues or metabolites of petasin.

The antibodies according to the present invention are produced with the aid of derivatives of petasin, which are preferably coupled to a carrier molecule. To our surprise, it was thus possible to avoid a production of antibodies directed against the coupling group of petasin or a potentially occurring modification of the immunodominant epitope situated in the vicinity of position 8.

The polyclonal or monoclonal antibodies are produced by immunization of mammals and/or birds by petasin or petasin derivatives of the general formula I



and obtained by means of the hybridome technique or recombinantly with the aid of antibody libraries.

Preferably the following derivatives coupled to a carrier molecule are used:

- Derivatives of petasin of the general formula I where the keto group in position 8 is replaced by a carboxyl group and coupled to a bovine serum albumin by means of EDAC.
- Derivatives of petasin of the general formula I where the keto group in position 8 is replaced by a carboxyl group and coupled to a bovine serum albumin or fibrinogen